Report No. 66206

MOBAY CHEMICAL CORPORATION
AGRICULTURAL DIVISION

Research and Development Department

TITLE: A Gas Chromatographic Method for the Determination of ®NEMACUR and Metabolites in Bovine Tissues and Milk.

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ABSTRACT: A gas chromatographic (GLC) method is described for the analysis of NEMACUR and metabolites in bovine tissues and milk. The method involves an initial extraction using acetone and dichloromethane followed by a partition cleanup and an oxidation of all the residues to the sulfone metabolites. An additional solvent partition cleanup was performed prior to GLC analysis for NEMACUR, NEMACUR sulfoxide and NEMACUR sulfone residues. Following GLC the residue was hydrolyzed and the resulting sulfone phenol methylated. GLC analysis for total NEMACUR residues accounted for NEMACUR, NEMACUR sulfoxide, NEMACUR sulfone, des-isopropyl NEMACUR, des-isopropyl NEMACUR sulfoxide and des-isopropyl NEMACUR sulfone. The sensitivity of the method is 0.01 ppm for tissues and 0.001 ppm for milk.

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NOTEBOOK REFERENCE: 78-R-150 and 75-159

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NEMACUR is a Reg. TM of the Parent Company of Farbenfabriken Bayer GmbH, Leverkusen.

Report No. 66206

A Gas Chromatographic Method for the Determination of NEMACUR and Metabolites in Bovine Tissue and Milk

INTRODUCTION

A gas chromatographic method has previously been developed for the analysis of NEMACUR, NEMACUR sulfoxide and NEMACUR sulfone in bovine tissues and milk.[1] A bovine metabolism study with NEMACUR sulfoxide-ring-UL-^{14}C, conducted later, showed that additional NEMACUR metabolites may be present in tissues and milk.[2] These metabolites were shown to be des-isopropyl NEMACUR and des-isopropyl NEMACUR sulfoxide.

This method was developed to include these additional metabolite residues. The original procedure was revised with several modification, i.e., dichloromethane was substituted for chloroform, sample was split prior to oxidation then combined and a solvent partition step was added using hexane and acetonitrile. NEMACUR, NEMACUR sulfoxide and NEMACUR sulfone residues as NEMACUR sulfone (expressed in NEMACUR equivalents) were then measured by GLC as in the original method (phosphorous mode). The des-isopropyl NEMACUR metabolites as des-isopropyl NEMACUR sulfone, would not gas chromatograph under these conditions; therefore, the sample residue was hydrolyzed, derivatized (TMAH) and the total residue (including all metabolites) measured as the methylated derivative of the sulfone phenol by GLC (sulfur mode, expressed in NEMACUR equivalents).

The extraction scheme used in this procedure has been shown to be adequate in extracting the des-isopropyl NEMACUR metabolites from tissue and milk.[2] Phenols which may exist in the sample would not be measured as they were not removed from the oxidizing media and therefore, not derivatized or detected.

DISCUSSION

A tentative method was submitted to Analytical Development Corporation (ADC), Monument, Colorado, entitled "A GLC Method for Total NEMACUR Residue in Animal Tissues and Milk," for validation and/or modifications to achieve satisfactory recovery of all metabolites. The final validation of this method is reported herein, pages 8-50.

A confirmatory method[1] has been reported earlier for NEMACUR, NEMACUR sulfoxide and NEMACUR sulfone residues (phosphorous mode) which utilized a different GLC column of different polarity, 5% OV-17 (primary column: 5% OV-210) and would be applicable in this method for this portion of the analysis. A confirmatory method for the second portion of this method (sulfur mode) utilized two different GLC columns of different polarity, 10% OV-3 for liver samples and 4.8% OV-17 + 6.2% QF-1 for samples other than liver (primary column: 5% OV-225).

Recovery experiments were run on tissues and milk and are shown in Tables I and II. Tissues were fortified at 0.05 ppm and 0.10 ppm. Milk was fortified at 0.005 ppm and 0.010 ppm. NEMACUR, NEMACUR sulfoxide, NEMACUR sulfone, des-isopropyl NEMACUR, des-isopropyl NEMACUR sulfoxide and des-isopropyl NEMACUR sulfone standards were used.

Recoveries in tissues ranged from 87-117% at 0.10 ppm and 73-158% at 0.05 ppm in the phosphorous mode of analysis. In the sulfur mode recoveries ranged from 84-138% at 0.10 ppm and 58-148% at 0.05 ppm. Recoveries in the sulfur confirmatory mode of analysis ranged from 82-106% at 0.10 ppm and 78-144% at 0.05 ppm.

Recoveries in milk ranged from 79-115% at 0.01 ppm and 61-68% at 0.05 ppm in the phosphorous mode of analysis. In the sulfur mode recoveries ranged from 84-128% at 0.01 ppm and 70-120% at 0.005 ppm. Recoveries in the sulfur confirmatory mode of analysis were 85-90% at 0.01 ppm fortification.

Typical control and recovery chromatograms (phosphorous mode) are shown in Figures 1-10 and for the sulfur mode Figures 11-20. Standard chromatograms for NEMACUR sulfone (phosphorous mode) and the methylated phenol sulfone (sulfur mode) for the primary and two confirmatory GLC columns are shown in Figures 21-27. A standard response curve for tissues and milk for the sulfur mode analysis is shown in Figures 28-29.

The instrumental sensitivity of the analytical residue method for tissues was 0.01 ppm (NEMACUR equivalents) and 0.001 ppm (NEMACUR equivalents) for milk. This was based on a minimum level of detectability of 1/10 the instrumental response for a 0.1 ppm standard (for tissues) and a 0.01 ppm standard (for milk) in which the standards were equal to or greater than 50% scale (12.5 cm on recorder chart) and that the base line noise level never exceeded 1/20 of the standard response. Day to day variations in instrumental response and control values, although very low, may affect this limit of sensitivity.

TABLE I

Recovery of NEMACUR and Metabolites in Tissues

Sample Type	Compound	Fortification Level (Ppm)	% Recovery Primary "P" Mode[1]	Primary "S" Mode[2]	Confirmatory "S" Mode[3]
Liver	NEMACUR	0.05	88	74	104
		0.10	90, 117, 89, 99	138, 120, 114, 110, 108	106
	NEMACUR Sulfoxide	0.05	73	76	
		0.10	87	111	
	NEMACUR Sulfone	0.05	73	72	
		0.10	93	112	
	Des-Isopropyl NEMACUR	0.05	-	80	
		0.10	-	109	
	Des-Isopropyl NEMACUR Sulfoxide	0.05	-	104	112
		0.10	-	105, 84, 109, 108	82
	Des-Isopropyl NEMACUR Sulfone	0.05	-	58	
		0.10	-	104	
Fat	NEMACUR	0.05	102	82	78
		0.10	90	96	
	NEMACUR Sulfoxide	0.05	104	98	
	NEMACUR Sulfone	0.05	112	94	
	Des-Isopropyl NEMACUR	0.05	-	110	
	Des-isopropyl NEMACUR Sulfoxide	0.05	-	70	86
		0.10		89	
	Des-Isopropyl NEMACUR Sulfone	0.05	-	72	
Kidney	NEMACUR	0.05	105	92	92
		0.10	110	115	
	NEMACUR Sulfoxide	0.05	96	86	
	NEMACUR Sulfone	0.05	103	88	
	Des-Isopropyl NEMACUR	0.05	-	116	
	Des-Isopropyl NEMACUR Sulfoxide	0.05	-	106	114
		0.10	-	115, 96	
	Des-Isopropyl NEMACUR Sulfone	0.05	-	88	
Muscle	NEMACUR	0.05	158, 118	128, 124	144, 120
		0.10	112	106	
	NEMACUR Sulfoxide	0.05	138, 108	98, 114	126
	NEMACUR Sulfone	0.05	148, 120	120, 124	124

Sample Type	Compound	Fortification Level (Ppm)	% Recovery Primary "P" Mode[1]	Primary "S" Mode[2]	Confirmatory "S" Mode[3]
Muscle	Des-Isopropyl NEMACUR	0.05		110	
	Des-Isopropyl NEMACUR } Sulfoxide }	{ 0.05 { 0.10		148 118	82
	Des-Isopropyl NEMACUR } Sulfone }	{ 0.05		138	

[1]GLC column: 5% OV-210 on Chromosorb W (HP).
[2]GLC column: 5% OV-225 on Chromosorb W (HP).
[3]GLC column: 4.8% OV-17 + 6.2% QF-1 on Gas Chrom Q (all tissues except liver).
10% OV-3 on Chromosorb W (HP) (liver tissues).

Control values were as follows:

	"P" Mode	Primary "S" Mode	Confirmatory "S" Mode
Liver	<0.01	<0.01	0.01
Fat	<0.01	<0.01	<0.01
Kidney	<0.01	<0.01	<0.01
Muscle	<0.01	<0.01	<0.01

For raw data and chromatograms, see Mobay Reports 66203 and 66205 (1978).

TABLE II

Recovery of NEMACUR and Metabolites in Milk

Sample Type	Compound	Fortification Level (Ppm)	% Recovery		
			Primary "P" Mode [1]	Primary "S" Mode [2]	Confirmatory "S" Mode [3]
Milk	NEMACUR	0.005	65	120	
		0.01	80, 90, 115, 79	84, 113, 108, 94	85
	NEMACUR Sulfoxide	0.005	61	88	
		0.01	84, 81	126, 112	88
	NEMACUR Sulfone	0.005	68	110	
		0.01	113, 92	128, 116	85
	Des-Isopropyl NEMACUR	0.005	-	80	
		0.01	-	124, 106	90
	Des-Isopropyl NEMACUR Sulfoxide	0.005	-	88	
		0.01	-	85, 90, 92	
	Des-Isopropyl NEMACUR Sulfone	0.005	-	70	
		0.01	-	119	

Control values were as follows:

	"P" Mode	Primary "S" Mode	Confirmatory "S" Mode
Milk:	< 0.001	<0.001	≤0.001

[1]GLC column: 5% OV-210 on Chromosorb W (HP).
[2]GLC Column: 5% OV-225 on Chromosorb W (HP).
[3]GLC Column: 4.8% OV-17 + 6.2% QF-1 on Gas Chrom Q.

For raw data and chromatograms, see Mobay Report 66204 and 66205 (1978).

REFERENCES

1. Thornton, J. S., "Determination of Residues of NEMACUR and its Metabolites in Plant and Animal Tissues," *J. Agr. Food Chem.*, 19, 890 (1971), Mobay Report No. 31143.

2. Gronberg, R. R., D. R. Flint and K. M. Pither, "The Metabolic Fate of NEMACUR Sulfoxide Administered Orally to a Lactating Dairy Cow," Mobay Report No. 41104 (1974).

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VERIFICATION REPORT

To: Chemagro Agricultural Divn.
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ADC Project: #415

Date: April 25, 1978

VERIFIED METHOD FOR THE DETERMINATION OF NEMACUR AND METABOLITES IN BOVINE TISSUES AND MILK

I. INTRODUCTION

The following is the method verified by Analytical Development Corporation for the determination of Nemacur and metabolites in bovine tissues and milk. It is based on Chemagro's report entitled "A GLC Method for Total Nemacur Residue in Animal Tissues & Milk", with modifications developed by Analytical Development Corporation (see Progress Reports No. 1 and No. 2 dated February 6, 1978 and April 25, 1978 respectively).

II. METHOD

A. Abstract

A specific gas chromatographic procedure for the determination of residues of Nemacur and metabolites in bovine matrices involves an acetone-dichloromethane extraction, partition cleanup, and a potassium permanganate oxidation of all compounds to the sulfone. An additional partition cleanup precedes gas chromatographic analysis in the phosphorous mode, where residues as Nemacur sulfone are detected (des-isopropyl metabolites are not detected at this point). Room temperature alcoholic hydrolysis converts all compounds to Nemacur phenol sulfone which is methylated with TMAH reagent and detected by gas chromatographic analysis in the sulfur mode (total residues detected in the sulfur mode). Using this method, the lower limit of detection is 0.1 ppm for tissues and 0.01 ppm for milk. The sensitivity limits are 0.01 ppm for tissues and 0.001 ppm for milk. Recoveries were run by fortification of matrices with specific compounds at the blending step. Recoveries at 0.1 ppm (tissues) and 0.01 ppm (milk) ranged from 80-120%. Recoveries at 0.05 ppm (tissues) and 0.005 ppm (milk) ranged from 50 - 150%.

B. Apparatus

1. Assorted laboratory glassware
2. Blender, Waring or equivalent

II. B. 3. Chromatographic columns, borosilicate glass

61 cm x 2 mm i.d. U-shaped
122 cm x 2 mm i.d. U-shaped (two)
91 cm x 2 mm i.d. U-shaped

4. Filter paper, Whatman 24.0 cm 2V fluted
" " , Whatman 11.0 cm No. 1

5. Gas chromatograph, Tracor Model 222 equipped with flame photometric detector (FPD), misting trap, and filters (phosphorous and sulfur) or equivalent

6. Maxi-mixer, Thermolyne or equivalent

7. N-Evap analytical evaporator, Organomation or equivalent

8. Rotary vacuum evaporator, all glass

9. Ultrasonic bath, Bransonic 220 or equivalent.

10. Water bath, 40°C

C. Reagents

1. Acetone - nanograde or equivalent

2. Acetonitrile - nanograde or equivalent

3. Benzene - nanograde or equivalent

4. Dichloromethane - nanograde or equivalent

5. Ethyl acetate - nanograde or equivalent

6. Hexane - nanograde or equivalent

7. Hydrochloric acid solution 3N, 245 ml concentrated HCl (reagent ACS) up to one liter with distilled H_2O

8. Hyflo Super-Cel - Johns-Manville Co.

9. Isopropyl alcohol - nanograde or equivalent

10. Magnesium sulfate, 20% w/v aqueous solution, 200 g magnesium sulfate up to 1 liter with distilled H_2O

II. C. 11. Mineral oil in hexane - 10% solution, 10 ml mineral oil (USP) up to 100 ml with hexane

12. Potassium hydroxide, 0.5N in isopropyl alcohol, 33 g KOH up to 1 liter with isopropyl alcohol

13. Potassium permanganate, 0.1M aqueous solution 15.8 g $KMnO_4$ up to 1 liter with distilled H_2O

14. Sodium sulfate, A.R., anhydrous granular

15. Trimethyl anilinium hydroxide (TMAH), 0.2N in methanol, Regis or Methelute, Pierce Chemical Co.

D. Standards

1. Stock Solutions

a. Nemacur 250 µg/ml - Dissolve 0.0250 g of recrystallized Nemacur in benzene and dilute to volume in a 100 ml volumetric flask.

b. Nemacur Sulfoxide - 250 µg/ml Nemacur equivalent - Dissolve 0.0263 g of recrystallized Nemacur sulfoxide in benzene and dilute to volume in a 100 ml volumetric flask.

c. Nemacur Sulfone 250 µg/ml Nemacur equivalent - Dissolve 0.0276 g of recrystallized Nemacur Sulfone in benzene and dilute to volume in a 100 ml volumetric flask.

d. Des-Isopropyl Nemacur 250 µg/ml Nemacur equivalent - Dissolve 0.0215 g of recrystallized Des-Isopropyl Nemacur in benzene and dilute to volume in a 100 ml volumetric flask.

e. Des-Isopropyl Nemacur Sulfoxide 250 µg/ml Nemacur equivalent - Dissolve 0.0229 g of recrystallized Des-Isopropyl Nemacur Sulfoxide in benzene and dilute to volume in a 100 ml volumetric flask (note: addition of 2 ml MeOH necessary to solubilize compound).

f. Des-Isopropyl Nemacur Sulfone 250 µg/ml Nemacur equivalent - Dissolve 0.0242 g of recrystallized Des-Isopropyl Nemacur Sulfone in benzene and dilute to volume in a 100 ml volumetric flask (note: addition of 2 ml MeOH necessary to solubilize compound).

II. D. 2. Fortification Solutions

a. Bovine Tissues - Pipet 2.5 ml of each stock solution into individual 250 ml volumetric flasks and dilute to volume with acetone. Each solution contains 2.5 µg/ml Nemacur equivalents.

0.5 ml of the solution represents 0.05 ppm
1.0 ml of the solution represents 0.10 ppm
1.5 ml of the solution represents 0.15 ppm
2.0 ml of the solution represents 0.20 ppm

b. Milk - Pipet 2.0 ml of each stock solution into individual 250 ml volumetric flasks and dilute to volume with acetone. Each solution contains 2.0 µg/ml Nemacur equivalents.

0.5 ml of the solution represents 0.005 ppm
1.0 ml of the solution represents 0.010 ppm
1.5 ml of the solution represents 0.015 ppm
2.0 ml of the solution represents 0.020 ppm

E. Procedure *

1. Extraction

a. Tissue Samples (other than fat)

(1) Weigh 25 grams of the homogenized sample into a blender jar.

(2) Add 250 ml of acetone and 15 grams of Super-Cel. Blend for three minutes at high speed.

(3) Filter with vacuum through Whatman No.1 filter paper in a Büchner funnel.

(4) Reblend the filter cake with 250 ml DCM plus 50 ml acetone for an additional two minutes and filter as above.

(5) Rinse the blender and filter cake with acetone.

(6) Transfer the combined filtrates to a one liter separatory funnel and shake for 30 seconds.

* NOTE: Throughout the procedure, any hexane or acetonitrile used is pre-saturated, one with the other.

II. E. 1. a. Tissue Samples (other than fat) cont.

(7) After layer separation (if any occurs), drain the lower phase through fluted filter paper containing 25 g Na_2SO_4 into a one liter flask.

(8) Evaporate the sample to dryness using a rotary evaporator at 40°C.

(9) Transfer the sample residue to a 500 ml separatory funnel with 250 ml of hexane.

(10) Rinse the flask with 150 ml acetonitrile and add the rinse to the separatory funnel.

(11) Shake the funnel for 30 seconds, allow the layers to separate, and drain the lower acetonitrile phase into a second separatory funnel containing 100 ml of hexane.

(12) Shake the second separatory funnel for 30 seconds. Allow phases to separate and drain the acetonitrile phase into a 500 ml flask.

(13) Repeat steps 11 & 12 with an additional 100 ml of acetonitrile each time.

(14) Divide each sample equally into two 250 ml flasks. Evaporate the extracts to dryness on a rotary vacuum evaporator at 40°C. Proceed to oxidation.

b. Fat Samples

(1) Weigh 25 grams of the chopped sample into a blender jar.

(2) Add 250 ml of hexane and 15 grams Super-Cel and blend at high speed for three minutes.

(3) Vacuum filter through filter paper in a Büchner funnel.

(4) Reblend the filter cake with 150 ml acetonitrile for an additional two minutes and filter as above.

II. E. 1. b. Fat Samples (cont.)

(5) Rinse the blender and filter cake with acetonitrile.

(6) Transfer the combined filtrates to a 500 ml separatory funnel using a few milliliters of acetonitrile to complete the transfer.

(7) Continue at step 11 under Tissue Samples.

c. Milk Samples

(1) Disperse the cream uniformly in the milk and weigh a 200 gram sample into a blender jar.

(2) Add 250 ml of acetone and 15 grams Super-Cel. Blend at high speed for two minutes.

(3) Vacuum filter through filter paper in a Büchner funnel.

(4) Reblend the filter cake with 250 ml DCM and 50 ml acetone for an additional 2 minutes and filter as above.

(5) Rinse the blender and filter cake with acetone.

(6) Transfer the combined filtrates to a one liter separatory funnel and shake for 30 seconds.

(7) After layer separation, drain the lower phase through fluted filter paper containing 25 g Na_2SO_4 into a one liter flask.

(8) Reduce the sample volume on a rotary evaporator.

(9) Re-extract the aqueous layer remaining after step 7 with 300 ml DCM plus 200 ml acetone. After layer separation, drain the lower phase through the same filter paper and Na_2SO_4 used in step 7 into the same one liter flask as previously used (containing the volume reduced in first extraction).

(10) Evaporate the combined extracts to dryness using a rotary evaporator at 40°C.

II. E. 1. c. Milk Samples (cont.)

(11) Dissolve the residue in 100 ml of hexane and transfer to a 250 ml separatory funnel.

(12) Rinse the flask with 100 ml of acetonitrile and add this to the separatory funnel.

(13) Shake the funnel 30 seconds, allow the layers to separate, and drain the lower acetonitrile phase into a second separatory funnel containing 50 ml of hexane.

(14) Shake the second separatory funnel for 30 seconds, allow the phases to separate, and drain the lower phase into a 250 ml flask.

(15) Repeat steps 13 and 14 with an additional 50 ml of acetonitrile.

(16) Divide each sample equally into two 250 ml flasks. Evaporate the extracts to dryness on a rotary vacuum evaporator at 40°C. Proceed to oxidation.

2. Oxidation (The sample was divided into halves to insure adequate oxidation capacity and to avoid any emulsion difficulties.)

Note: Start 0.05 ppm, 0.1 ppm, 0.15 ppm, and 0.2 ppm Nemacur standards for tissues or start 0.005 ppm, 0.01 ppm, 0.015 ppm, and 0.02 ppm Nemacur standards for milk. Each standard is run in only one flask. Add an appropriate volume of acetone to each to bring up to a total of 2 ml.

a. Dissolve the sample residue in 2 ml acetone.

b. Add 5 ml magnesium sulfate solution and 20 ml potassium permanganate solution to each, rinsing down the sides of the flask during additions.

c. Mix and let stand 15 minutes, swirling approximately every 5 minutes.

d. Transfer to a 125 ml separatory funnel using 25 ml DCM to complete the transfer (sonicate to remove residue).

II. E. 2. Oxidation (cont.)

e. Shake the separatory funnel 30 seconds, allow the phases to separate (centrifuge if necessary) and drain the lower phase of each sample half through ~~fluted filter paper~~ containing 25 g Na_2SO_4 into a common 250 ml flask.

f. Repeat DCM extraction two additional times with 25 ml DCM.

g. After the final extraction, wash the sodium sulfate with 10 ml DCM.

h. Evaporate the combined extracts just to dryness on a rotary evaporator at 40°C.

3. Cleanup Partition

a. Dissolve residue from section 2.h. in 50 ml hexane and transfer to a 125 ml separatory funnel.

b. Rinse the flask with 50 ml acetonitrile and transfer to the separatory funnel.

c. Shake the separatory funnel 30 seconds and allow the phases to separate.

d. Drain the lower acetonitrile phase back into the 250 ml flask and discard the hexane rinse.

e. Transfer the acetonitrile back into the separatory funnel and repeat the hexane partition twice with 50 ml hexane.

f. After the final partition, drain the acetonitrile into a clean 250 ml flask and evaporate just to dryness on a rotary evaporator.

4. Gas Chromatographic Analysis "A" (Phosphorous Mode)

a. Dissolve the residue from section 3.f. in 1.0 ml of ethyl acetate.

b. Inject 5 µl of sample and 5 µl of 0.1 ppm oxidized Nemacur standard (tissues) or 5 µl 0.01 ppm oxidized Nemacur standard (milk) [See section 8.b. (Gas Chromatographic Parameters "A")].

II. E. 4. Gas Chromatographic Analysis "A" (Phosphorous Mode) cont.

c. Identify Nemacur Sulfone peak by its retention time.

d. Calculations

(1) Nemacur, Nemacur Sulfoxide, and Nemacur Sulfone residues as Nemacur equivalents. Calculate the ppm Nemacur equivalents present in a sample by comparing the response for an unknown to the response for a known amount of Nemacur standard carried through the procedure from the oxidation step.

$$\text{ppm} = \frac{(\text{ppm standard}) \times (\text{pk. ht. sample})}{\text{pk. ht. standard}} \times \text{dilution factor}$$

(2) Recoveries are calculated as follows:

$$\% \text{ Recovery} = \frac{\text{ppm Found}}{\text{ppm Added}} \times 100$$

e. Evaporate the ethyl acetate to dryness and proceed to hydrolysis.

5. Hydrolysis

a. Add 10 ml benzene to the residue from step 4.e.

b. Add 10 ml 0.5N KOH in isopropyl alcohol.

c. Mix well and let stand 30 minutes, swirling approximately every 10 minutes.

d. Transfer the mixture to a 125 ml separatory funnel.

e. Complete the transfer by rinsing the flask with 10 ml isopropyl alcohol and 20 ml H_2O.

f. Add 50 ml benzene to the separatory funnel and shake for 30 seconds. Allow the phases to separate and drain the lower aqueous phase into a 250 ml separatory funnel.

g. Add 5 ml of H_2O to the first separatory funnel and shake for 30 seconds. Drain the lower phase into the 250 ml separatory funnel.

II. E. 5. Hydrolysis (cont.)

h. Add 5 ml 3N HCl to the 250 ml separatory funnel.

i. Add 50 ml DCM, shake 30 seconds, allow phases to separate, and drain lower phase through 25 g Na_2SO_4 in fluted filter paper into a 250 ml flask.

j. Repeat step i twice more using 25 ml portions of DCM.

k. Rinse Na_2SO_4 with 15 ml DCM.

l. Add 100 µl 10% mineral oil in hexane solution to each flask.

m. Evaporate to dryness on a rotary evaporator.

n. Transfer the residue from the flask into a 15 ml centrifuge tube with three 2 ml portions of acetone using a Pasteur pipet. Evaporate to dryness on an N-Evap evaporator.

o. Add 0.5 ml TMAH and cap with aluminum foil lined caps. Agitate the tube to wet the wall thoroughly to ensure the sample is completely dissolved in TMAH reagent. Continue to gas chromatographic analysis "B". Note that any dilutions for samples exceeding the standard curve plot must be made with TMAH.

6. Gas Chromatographic Analysis "B" (Sulfur Mode)

a. Inject 15 µl of sample and 15 µl of the standards [See section 8.c. (Gas Chromatographic Parameters "B")].

b. Identify the methylated ϕ-SO_2 by its retention time.

c. Calculations

(1) Nemacur, Nemacur Sulfoxide, Nemacur Sulfone, Des-isopropyl Nemacur, Des-isopropyl Nemacur Sulfoxide, Des-isopropyl Nemacur Sulfone as Nemacur equivalents. On 2 x 2 cycle logarithmic paper, a plot is made of standard peak heights vs. parts per million. The peak height

II. E. 6. c. Calculations

(1) cont.

of the sample is located on the curve and the corresponding ppm obtained and reported in Nemacur equivalents after dilution factors are taken into account.

ppm found on curve x dilution factor

(2) Recoveries are calculated as follows:

$$\% \text{ Recovery} = \frac{\text{ppm found}}{\text{ppm added}} \times 100$$

7. Gas Chromatographic Analysis "C" or "D" (Sulfur Mode Confirmatory Columns)

a. Proceed as for Gas Chromatographic Analysis "B" [see sections 8.d. and 8.e. (Gas Chromatographic Parameters "C" or "D")]. Use the 3' 11% OV-17 + QF-1 column as a confirmatory column for all matrices except liver. Use the 4' 10% OV-3 column as a confirmatory column for liver matrix samples.

8. Gas Chromatographic Analysis Parameters

a. Instrument: Tracor Model 222 equipped with a flame photometric detector (phosphorous and sulfur filters)

b. Column "A" (Phosphorous Mode)

(1) 61 cm x 2 mm i.d. U-shaped borosilicate glass packed with 5% OV-210 on 80/100 mesh Chromosorb W, H.P.

(2) Column Conditioning

(a) Purge 30 minutes with helium.
(b) No flow condition for two hours at 250°C.
(c) Flow condition at least two hours at 250°C with helium flow at 30 cc/min.

(3) Instrument Conditions

(a) Injection port temperature: 250°C
(b) Detector temperature: 250°C

II. E. 8. b. (3) Instrument Conditions (cont.)

(c) Column oven temperature: 220°C
(d) Nitrogen carrier flow: 83 ml/min
(e) Air flow: 375 ml/min
(f) Oxygen flow: 42 ml/min
(g) Hydrogen flow: Adjust flow so the lower limit of detection shows a 1/2 scale peak height.
(h) Chart speed: 0.25 inch/min
(i) Attenuation: 512 x 10^2

c. Column "B" (Sulfur Mode)

(1) 122 cm x 2 mm i.d. U-shaped borosilicate glass packed with 5% OV-225 on 80/100 mesh Chromosorb W, H.P.

(2) Column Conditioning

(a) Purge 30 minutes with helium.
(b) No flow condition for 7 hours at 250°C.
(c) Flow condition overnight at 225°C with helium flow at 30 cc/min.

(3) Instrument Conditions

(a) Injection port temperature: 240°C
(b) Detector temperature: 240°C
(c) Column oven temperature: 210°C
(d) Nitrogen carrier flow: 76 ml/min
(e) Air flow: 375 ml/min
(f) Oxygen flow: 14 ml/min
(g) Hydrogen flow: Adjust flow so the lower limit of detection shows a 1/2 scale peak height.
(h) Chart speed: 0.25 inch/min
(i) Attenuation: 1024 x 10

d. Column "C" (Sulfur Mode Confirmatory Column - all matrices except liver)

(1) 91 cm x 2 mm i.d. U-shaped borosilicate glass packed with 11% OV-17 + QF-1 on 80/100 mesh Gas Chrom Q.

(2) Column Conditioning (same as Column "B")

II. E. 8. d. (3) Instrument Conditions

(a) Injection port temperature: 240°C
(b) Detector temperature: 235°C
(c) Column oven temperature: 180°C
(d) Nitrogen carrier flow: 89 ml/min
(e) Air flow: 375 ml/min
(f) Oxygen flow: 5 ml/min
(g) Hydrogen flow: Adjust flow so the lower limit of detection shows a 1/2 scale peak height.
(h) Chart speed: 0.5 inch/min
(i) Attenuation: 1024×10^2

e. Column "D" (Sulfur Mode Confirmatory Column for liver matrix samples)

(1) 122 cm x 2 mm i.d. U-shaped borosilicate glass packed with 10% OV-3 on 80/100 mesh Chromosorb W, H.P.

(2) Column Conditioning (same as Column "B")

(3) Instrument Conditions

(a) Injection port temperature: 240°C
(b) Detector temperature: 235°C
(c) Column oven temperature: 190°C
(d) Nitrogen carrier flow: 89 ml/min
(e) Air flow: 375 ml/min
(f) Oxygen flow: 5 ml/min
(g) Hydrogen flow: Adjust flow so the lower limit of detection shows a 1/2 scale peak height.
(h) Chart speed: 0.25 inch/min
(i) Attenuation: 256×10^2